EXHIBIT E

to 8.46 copies/cell). Only three the cases for which CGH detected a gain at 8q had a *MYC* gain according to PCR. PCR detected a decrease in *MYC* gene copy number in six tumors, one of which also showed loss of genetic material at 8q24 by CGH.

The ratio of *NKX3.1* to *MYC* was below the TI in 22 cases (63%), comprising distinct classes of genetic alterations: loss of *NKX3.1* and normal *MYC* (8 tumors), normal *NKX3.1* and gain of *MYC* (4 tumors), or loss of *NKX3.1* concurrent with gain of *MYC* (7 tumors). CGH did not detect concurrent 8p loss and 8q gain in any of the seven tumors. Conversely, two tumors with concurrent loss at 8p and gain at 8q according to CGH were not detected by PCR. Four tumors exhibited loss of both genes or gain of both genes, leading to a significant decrease in the ratio of *NKX3.1* to *MYC* below normal in three cases. Our findings concur with recent observations of an association between loss of genetic material at 8p and gain at 8q in prostate cancer *(15, 16)*.

In summary, our relative real-time PCR quantification method based on the LightCycler system differs from previous methods in that it uses the fluorescent dye SYBR Green I, which rapidly and sensitively detects low amounts of DNA molecules. Compared with hybridization probes, SYBR Green I is less expensive, which becomes particularly important when screening of larger series of tissue samples is intended. As a modification of the $2^{-\Delta\Delta CT}$ method, we generated internal calibration curves to determine the gene-specific PCR efficiencies for each individual run. This approach allows for screening analysis of individual genes in separate runs under optimum PCR conditions. As demonstrated for prostate cancer samples, our real-time PCR quantification method is suitable for screening of specific genes and could be suitable for other applications in which gene copy number alterations are an important feature of pathogenesis.

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Treatment of Maternal Blood Samples with Formaldehyde Does Not Alter the Proportion of Circulatory Fetal Nucleic Acids (DNA and mRNA) in Maternal Plasma, *Satheesh Kumar Reddy Chinnapapagari, Wolfgang Holzgreve, Olav Lapaire, Bernhard Zimmermann, and Sinuhe Hahn** (University Women's Hospital/Department of Research, Basel, Switzerland; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, Spitalstrasse 21, CH 4031 Basel, Switzerland; fax 41-61-265-9399, e-mail shahn@uhbs.ch)

Cell-free fetal DNA and fetal mRNA can be found in maternal plasma and used for noninvasive prenatal diagnosis and, potentially, for monitoring and prognosis of certain pregnancy-related clinical conditions *(1–12)*. The excess of maternal DNA in these samples, however, complicates the detection of fetal genetic traits that are similar to those in the maternal genome (e.g., point mutations) *(1, 13, 14)*. In normal pregnancies, fetal DNA represents only ~3–6% of the total DNA in maternal plasma *(15)*. Thus, technical challenges lie in either developing methods permitting the reliable differentiation of fetal genetic loci or in reducing the amount of circulatory maternal DNA.

Dhallan et al. *(16)* have recently reported that the addition of formaldehyde to maternal blood samples increases the proportion of cell-free fetal DNA in maternal plasma by decreasing the concentration of maternal DNA. This effect was proposed to reflect an ability of formalde-

hyde to stabilize the maternal blood cells, thereby preventing the release of DNA from these cells should they die during sample collection and processing.

We have sought to verify this report and have investigated whether the addition of formaldehyde to maternal blood samples does indeed significantly alter the proportion of fetal DNA in maternal plasma samples. We also examined whether such treatment would improve the yield of fetal RNA. The rationale for this additional analysis was provided by the recent observation that circulating fetal hematopoietic cells may contribute to the pool of mRNA molecules in plasma *(17)* and the view that formaldehyde treatment may inhibit the activity of any ribonucleases in the sample.

We also examined whether the effect mediated by formaldehyde described by Dhallan et al. *(16)* was specific for the centrifugation conditions that they used or whether this would also be applicable for centrifugation conditions routinely used in our laboratory and in many other institutions *(18, 19)*. These experimental protocols can therefore be summarized as follows:

A. Method exactly as described by Dhallan et al. *(16)* (formaldehyde treatment)
B. Method described by Dhallan et al. minus formaldehyde
C. Our routine centrifugation procedure plus the addition of formaldehyde
D. Our routine centrifugation procedure (without formaldehyde)

After obtaining approval from the Cantonal Institutional Review Board of Basel, Switzerland, we collected 18 mL of peripheral blood from pregnant women (n = 26; median gestational age, 15 weeks; range, 11–40 weeks), who had all given written informed consent. All samples were processed within 1 h of venipuncture, and all analyses were performed in a blinded manner. The maternal blood samples were collected in two separate 9-mL EDTA tubes (Sarstedt), one of which was modified as described by Dhallan et al. *(16)* in that it contained 0.225 mL of a 10% neutral-buffered solution containing formaldehyde (40 g/L; Sigma); the other was not modified. After venipuncture, the contents of each of these two tubes were split into two fractions. One part was examined by protocols A and B, being centrifuged as described by Dhallan et al. (200*g* for 10 min, 1600*g* for 10 min, 1600*g* for 10 min) with brake and acceleration settings set to zero *(16)*. The other two fractions, to be examined by protocols C and D, were centrifuged by our standard protocol (1600*g* for 10 min at 4 °C and 16 000*g* for 10 min at room temperature) *(18, 19)*. In each case, DNA was extracted from 400 μL of plasma by use of the High Pure PCR Template Preparation Kit (Roche Diagnostics) and eluted in 100 μL of elution buffer. In a similar manner, total RNA was extracted from 800 μL of plasma as described by Ng et al. *(6)*, with use of an RNeasy mini column (RNeasy Mini Kit; Qiagen) and processed according to the manufacturer's instructions. RNA was eluted with 30 μL of RNase-free water, aliquoted, and stored at −80 °C. The samples were treated with DNase to remove any contaminating DNA (RNase-Free DNase Set; Qiagen). The concentration and integrity of total RNA were monitored by use of an Agilent 2100 Bioanalyzer (Agilent Technologies) with the RNA 6000 Nano/Pico Lab-on-a-chip reagent set. The RNA 6000 Ladder used was a set of six RNA transcripts with lengths of 0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 kb from Ambion.

Total DNA in the plasma was quantified by a TaqMan® real-time PCR assay (Applied Biosystems) for a chromosome 21-specific sequence *(20)*. The amplification primers were 5′-CCCAGGAAGGAAGTCTGTACCC-3′ (forward) and 5′-CCCTTGCTCATTGCGCTG-3′ (reverse), and the dual-labeled fluorescent probe was 5′-(FAM)CTGGCTGAGCCATC(MGB)-3′, where FAM is 6-carboxyfluorescein and MGB is minor groove binding. Fetal DNA in the sample was quantified by real-time PCR for a Y-chromosome-specific sequence as described previously *(5)*.

Total mRNA in the plasma was quantified by a TaqMan one-step real-time quantitative reverse transcription-PCR (RT-PCR; Applied Biosystems) using a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific assay. The amplification primers were 5′-CCACATCGCTCAGACACCAT-3′ (forward) and 5′-ACCAGGCGCCCAATACG-3′ (reverse), and the labeled probe was 5′-(VIC)CCAAATCCGTTGACTCCGACCTTCAC(TAMRA)-3′ (VIC is an ABI registered trademark and TAMRA is 6-carboxytetramethylrhodamine). Placentally derived fetal mRNA in the plasma was quantified by one-step real-time quantitative RT-PCR using an assay for corticotropin-releasing hormone (*CRH*) as described previously *(6)*.

The real-time and RT-PCR reactions were set up according to the manufacturer's instructions in a reaction volume of 25 μL. Each sample was analyzed in triplicate. For the RT-PCR assay, multiple negative water blanks were included in every analysis. A calibration curve for the quantification of *GAPDH* mRNA was prepared with serial dilutions of human control RNA (ranging from 15 to 0.23 pg), according to the manufacturer's instructions (PE Applied Biosystems) and using their estimation that 1 pg of the control RNA contains ~100 copies of *GAPDH* transcript. The RT-PCR assay was carried out by initiating the reaction at 50 °C for 2 min, followed by a reverse transcription step at 48 °C for 30 min. After a 5-min denaturation at 95 °C, the real-time PCR was carried out with 45 of the following cycles: a denaturation step of 94 °C for 15 s and an annealing/extension step of 60 °C for 1 min. All statistical analyses were performed with Sigma Stat software (SPSS), using the paired Student *t*-test.

The concentration of total DNA was not significantly ($P = 0.23$) altered by the formaldehyde treatment with the centrifugation protocol of Dhallan et al. *(16)* (protocols A and B: means = 3248 and 3643 genome-equivalents/mL of plasma, respectively) or with the centrifugation protocol used in our laboratory (protocols C and D: means = 3288 and 3157 genome-equivalents/mL, respectively; $P = 0.75$; Table 1 and Fig. 1A).

Fetal DNA (*SRY*) concentrations were unaffected by

Table 1. Concentrations and proportions of total cell-free DNA and fetal DNA in maternal plasma samples prepared by four different protocols.

	Protocol[a]			
	A	B	C	D
Total DNA,[b] GE/mL				
Mean (median)	3248 (2352)	3643 (3318)	3288 (2800)	3157 (2718)
Range	1212–3248	1385–6382	1113–8071	1312–8090
Fetal DNA,[b] GE/mL				
Mean (median)	89 (55.5)	97 (89.5)	87 (65.5)	82 (59)
Range	22–159	24–241	26–186	34–227
Fetal DNA, %				
Mean (median)	2.6 (2.6)	2.6 (2.4)	2.7 (2.8)	2.6 (2.8)
Range	1.19–4.33	1.0–4.43	26–186	0.90–4.11

[a] Protocol A: Dhallan et al. *(16)* protocol with formaldehyde treatment. Protocol B: Dhallan et al.*(16)* protocol without formaldehyde treatment. Protocol C: our routine protocol with formaldehyde treatment. Protocol D: our routine protocol without formaldehyde treatment.

[b] Cell-free DNA concentrations are given in genome-equivalents (GE)/mL of maternal plasma. (see the online Data Supplement for the complete data set).

formaldehyde treatment as described by Dhallan et al. (Table 1 and Fig. 1B). Consequently, we were not able to discern any effect of formaldehyde on the proportion of fetal DNA (Table 1 and Fig. 1C). For the complete data set, see the Data Supplement that accompanies the online versionofthisTechnicalBriefathttp://www.clinchem.org/content/vol51/issue3/.

Formaldehyde treatment did not significantly change the proportion of fetal CRH mRNA and maternal mRNA concentrations in maternal plasma (median proportions of fetal *CRH* mRNA for protocols A, B, C, and D were 3.5%, 3.2%, 2.9%, and 3.5%, respectively).

Our data therefore do not support the report by Dhallan et al. *(16)*, which stated that formaldehyde treatment led to an increase in the proportion of cell-free fetal DNA in maternal plasma samples of 20–50% or more. The reason for this difference is unclear at present, but it may be attributable to the use of two different approaches to quantifying the proportions of fetal DNA in the maternal plasma samples. Considerable variation in this proportion may exist even when using a standard real-time PCR protocol, as is evident from the recently concluded large-scale National Institute of Child Health and Human Development-funded "NIFTY" study, in which consider-

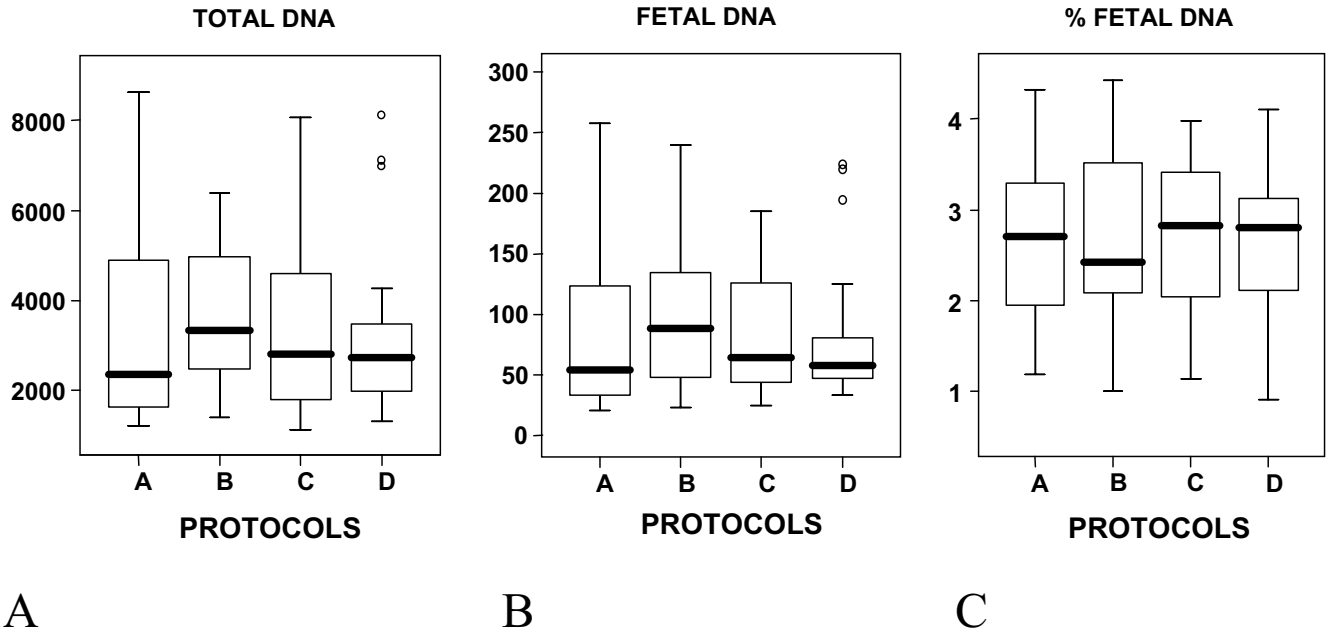

Fig. 1. Concentrations and proportions of total cell-free DNA and fetal DNA in maternal plasma samples prepared by four different protocols.

Total cell-free DNA (chromosome 21 locus; *A*), as well as cell-free fetal DNA (*SRY*; *B*) concentrations were determined by real-time PCR and are represented as genome-equivalents/mL of maternal plasma. The corresponding percentages of fetal DNA are also plotted (*C*). *Protocols A* and *B* refer to the processing protocol of Dhallan et al. *(16)* with and without formaldehyde, respectively. *Protocols C* and *D* refer to our routine laboratory protocol with and without formaldehyde, respectively. Box plots show the median (*line inside* the *box*) and 75th and 25th percentiles (*limits* of *box*). The *upper* and *lower error bars* indicate the 10th and 90th percentiles, respectively. Outliers are indicated by ○.

able variations in detection sensitivity and quantification of fetal DNA concentrations were noted among laboratories very familiar with real-time PCR technology *(21)*.

Further studies will be needed to clarify this issue in addition to the continued exploration of other strategies for the investigation of complex fetal genetic traits by analysis of maternal plasma. These strategies may include the enrichment of fetal sequences by size-fractionation of plasma DNA *(22, 23)* or the use of mass spectroscopy *(24)*, which has recently been shown to permit reliable detection of fetal point mutations.

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Lack of Dramatic Enrichment of Fetal DNA in Maternal Plasma by Formaldehyde Treatment, *Grace T.Y. Chung,[1†] Rossa W.K. Chiu,[1†] K.C. Allen Chan,[1] Tze K. Lau,[2] Tse N. Leung,[2] and Y.M. Dennis Lo[1*]* (Departments of [1] Chemical Pathology and [2] Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; † these authors contributed equally; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong Special Administrative Region, China; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

The discovery of fetal DNA in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis *(1–6)*. A recent report *(7)* indicated that the proportion of fetal DNA in maternal plasma can be dramatically enriched through the adoption of a blood-processing protocol involving the addition of formaldehyde to maternal blood samples. Dhallan et al. *(7)* suggested that these observations might be the result of several factors, including a reduction in background maternal DNA by minimization of maternal cell lysis through formaldehyde-mediated cell membrane stabilization and the use of a gentle centrifugation protocol, as well as the preservation of fetal DNA through nuclease inhibition by formaldehyde.

In view of the profound implications of the study *(7)*, we aimed to validate and investigate the underlying mechanisms of the reported phenomenon. To assess the effects of several contributory factors, our study was conducted in three successive stages. In the first part of the study, we aimed to verify the effects of the previously published protocol *(7)* on total DNA concentrations in plasma from nonpregnant individuals. In the second part of the study, we evaluated the effects of formaldehyde addition on total and fetal DNA concentrations in maternal plasma in relation to the time of blood processing (0, 6, and 24 h after blood collection). In the last part of the study, we investigated whether the reported enrichment in circulating fetal DNA concentrations *(7)* might be a consequence of the imprecision of the analytical method chosen by the authors.

All participants were recruited with informed consent